SUBTASK 2.9 – FRACTIONATION OF ORGANICS FROM AIR PARTICULATES WITH SUBCRITICAL WATER

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DISCLAIMER

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ABSTRACT

Although there are a vast number of studies on particulate matter (PM), a significant portion, mainly polar PM constituents, still remains unidentified. Consequently, toxicological studies are limited when relating adverse health effects to known components. Therefore, we have employed subcritical water fractionation, which allows for extractions of neglected polar compounds. Instead of employing analytical methods, which have to be targeted on species of interest, the importance of a wide-polarity range of fractions was evaluated using toxicological assays. Two in vitro assays evaluating oxidative stress and genotoxicity caused by PM were adopted to approximate possible adverse health effects to the human respiration system.

Different PM samples, such as diesel exhaust, wood smoke, and ambient Pittsburgh influenced by coke emissions, induced oxidative stress in murine macrophages (immune cells). Comparable results were also obtained for human bronchial epithelial cells. As hypothesized, the results demonstrated a significant contribution from polar and midpolar fractions. The oxidative stress caused by wood smoke PM fractions was linked to oxygenated polyaromatics.

The comparison of subcritical water fractions from two ambient Pittsburgh PM samples (with and without coke emission influence) showed that various polarity fractions of both PM samples exhibited genotoxicity, suggesting the different constituents in those samples were responsible for genotoxicity. Evaluation of direct genotoxicity of two diesel exhaust PM samples 1) generated as a reference and 2) generated in the presence of an oxidizer, showed genotoxicity in the presence of the oxidizer was higher.

In summary, the data generated confirm our hypothesis showing the toxicological importance of typically neglected polar PM fractions. In addition, we have demonstrated that even samples of slightly different origin such as two diesel PM or ambient PM samples from Pittsburgh may have different toxicological impact. Thus the conditions at which PM was generated can significantly influence its toxicity.

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EXECUTIVE SUMMARY

Although there are a vast number of studies on particulate matter (PM), a significant portion of PM constituents still remains unidentified. Consequently, toxicological studies are limited when relating adverse health effects to known components. In previous work, subcritical water was employed to obtain a wide-polarity range of fractions from PM. The use of subcritical water has enabled us to extract both polar (neglected by organic solvents) and nonpolar species. The importance of the fractions was initially evaluated using a cytotoxicity test, showing a significant contribution from polar fractions. To validate initial data, an assay more closely approximating the human respiratory system was adopted. A genotoxicity assay was implemented as a marker of DNA damage.

In this study, we have measured an oxidative stress on the basis of glutathione (GSH) upregulation/depletion caused by fractions of diesel exhaust and wood smoke PM and ambient Pittsburgh PM influenced by coke emissions using macrophages (immune cells). Initial tests also confirmed the feasibility and potential of human bronchial epithelial cells. The results demonstrated a significant contribution from polar and midpolar fractions. The oxidative stress caused by wood smoke PM fractions was evaluated with respect to their components, suggesting oxygenated polyaromatics may be significant contributors.

The fractionation method and genotoxicity assay were also applied to three ambient PM samples obtained from Pittsburgh. Two ambient Pittsburgh PM samples (with and without coke emissions influence) were genotoxic. Different-polarity fractions of ambient PM and PM influenced by coke emissions exhibited genotoxicity, suggesting the different constituents in those PM samples were responsible for genotoxicity. PM collected in a Pittsburgh highway tunnel did not exhibit direct genotoxicity.

We have also compared direct genotoxicity of two diesel exhaust PM samples: 1) generated as a reference and 2) generated in the presence of an oxidizer. Interestingly, the genotoxicity in the presence of the oxidizer was higher. As demonstrated in previous work, less studied polar fractions are significant contributors to diesel exhaust PM genotoxicity.

In summary, the data generated confirmed our hypothesis showing the toxicological importance of typically neglected polar PM fractions.

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BACKGROUND

Particulate Matter Toxicology

Compelling epidemiological and toxicological studies link atmospheric PM to pulmonary disease, including asthma.[1–4] In the presence of different types of PM (ambient, diesel, fly ash), both in vivo and in vitro studies show increased oxidative stress, suggesting it is an important mechanism of PM-induced lung inflammation. [5–14]

PM may exert oxidative stress on cells in the lung by exposing or stimulating the cells to produce reactive oxygen and nitrogen species (ROS/RNS).[4–8,10,12,15] Among the cells participating in the immune response are alveolar and bronchial epithelial cells and alveolar macrophages.[5–9,15–21] A model of respiratory system exposure to PM shows ca. 50% of fine particulate is retained on tracheal and bronchial epithelial cells, and an additional 20% of total fine particulate reaches alveoli with macrophages.[22] The redox balance of the cells is governed by a well-balanced system of enzymes with an ongoing number of cyclic reactions, such as generation of ROS and RNS, oxidation of glutathione (GSH), reduction of glutathione dimer (GSSG), etc.[5,7,18,21,23–27] The PM in the airways can activate inflammatory cells to produce ROS and may also participate in the direct generation of ROS and RNS.

Besides studies on pulmonary disease, there is a relationship between chronic exposure to high PM levels and cancer. [1,28] There are a number of mutagenic studies on PM showing the mutagenic effect of polycyclic aromatic hydrocarbons (PAHs).[8,11,29–39] The majority of mutagenicity studies employ bacterial assays using *Salmonella* or *Escherichia coli* strains, which were shown to give a response for chemicals defined by the International Agency for Research on Cancer (IARC) as mutagenic.[40–43]

Fractionation in Toxicological Studies

Epidemiological and inhalation studies show that PM toxicity is not only related to PM size but also to chemical composition.[1,4] The knowledge of the PM health impact grows proportionally with the development of analytical and toxicological techniques and the links between them. Currently, PM toxicity is mainly associated with inorganic species and nonpolar organics. Only recently has a possible contribution from polar organics been recognized.[44,45] Nevertheless, because of difficulty in routinely purifying and analyzing polar compounds, toxicological studies showing the importance of polar fractions did not identify their constituents. To evaluate toxicity of different PM components, the fractionation with a series of organic solvents was employed in both instillation and in vitro PM studies.[44–51] The solvent extractions/fractionations are limited by the polarity of the employed solvent.[52] Frequently, a lower-polarity solvent (such as methylene chloride) is used for the recovery of a crude extract, while the fractionation is performed using a solvent of stronger polarity such as methanol.[45,47,51,53–55]

Subcritical water has been shown to extract a wide-polarity range of organics. The advantage of water is its ability to extract polars at lower (25°–150°C) temperatures and nonpolars at higher (e.g., 250°C) temperatures. Therefore, in our previous work, we have employed subcritical (hot pressurized liquid) water for selective extraction of wood smoke and diesel exhaust PM. We have demonstrated that the variety of organics was selectively extracted from wood smoke PM. Polar compounds such as methoxyphenols (syringols and guaiacols) were found extracted at 25°C, oxyPAHs were extracted from 100° to 150°C, and nonpolar PAHs were extracted at temperatures above 150°C.[56, 57]

The aim of this project was to evaluate the toxicological significance of polar and nonpolar PM components in a system more closely approximating the human respiration system. In addition, different types of PM were studied.

EXPERIMENTAL

Sample Material

Bulk diesel exhaust PM was collected from the exhaust pipe of a diesel bus, homogenized, and stored frozen until use. Bulk wood smoke PM was collected from a chimney that vented an airtight wood stove burning a mix of hardwoods. Fifty-milligram portions of each material were used for all extraction experiments.[56, 57]

Instrumentation

The hot pressurized extraction apparatus, the equipment for solvent extraction, and analytical instrumentation such as a gas chromatography mass spectrometer (GC/MS) are described in the manuscript to be published in Environmental Toxicology and Chemistry.[57]

Toxicity Assays

Toxicity tests were performed on combined water fractions and individual water fractions. Toxicity tests were performed on nitrogen-dried extracts redissolved in dimethyl sulfoxide (DMSO) and Hank's balanced salt solution (HBSS) (Fisher Scientific, Pittsburgh, PA, USA) adjusted to pH ~7. DMSO was employed to transfer PM into aqueous suspension. DMSO may act as a radical scavenger; therefore, it may affect PM toxicity.[7] Thus we limited the concentrations of DMSO to 1% (v/v) in the toxicological assays. No effects of DMSO (1% v/v) were observed. We also verified that the dried organic solvent residues (solvent blanks) did not exhibit a toxic response.

Oxidative Stress Test

The potential oxidative stress was evaluated on the basis of GSH depletion and upregulation by exposing murine macrophages and human bronchial epithelial cells to PM fractions (12 h). Murine macrophages (RAW 264.7) and human bronchial epithelial cells (BEAS-2B) were purchased from American Type Culture Collection (Manassas, VA, USA). The

RAW 264.7 cells were grown in tissue culture flasks (75 mL) in Dulbecco's modified Eagle media (Fisher Scientific, Pittsburgh, PA, USA) with 4 mM glutamine, 1.5 g $NaHCO_3$, 4.5 g/L glucose, 10% (v/v) fetal calf serum, 1% (v/v) penicillin, and 1% (v/v) streptomycin in a 37°C incubator with a 95% O_2, 5% CO_2 atmosphere. The BEAS-2B cells were grown in Collagen I precoated tissue culture flasks (ca. 5 µL per 1 cm^2) in Lechner and LaVeck medium-9 **(**LHC-9) with supplements and growth factors (BPE, hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B and retinoic acid) (Clonetics, Walkersville, MD, USA). To transfer cells onto microplates, the cells were harvested with trypsin (0.05%) and 53 mM EDTA in HBSS buffer, centrifuged at low speed (1000 rpm for 5 min), and resuspended in fresh medium without serum and phenol red.

The oxidative stress tests were performed on four independent days (each time in quadruplicate) in clear bottom, white polystyrene 96-well microplates. The microplate for BEAS-2B cells as also precoated with Collagen I. The results were evaluated employing a two-way analysis of variance (ANOVA), for $P < 0.05$. On day one, culture cells were plated to each well, 180 µL of 10^6/mL of RAW 264.7 cells or 180 µL of 10^5/mL of BEAS-2B. After 24 h (48 h for BEAS-2B), 20 µL of sample was added. The oxidative stress via GSH depletion/upregulation was determined after 12 h exposure. Parallel to GSH depletion, cytotoxicity was monitored using lactate dehydrogenase (LDH). Intracellular GSH determination was performed with a monochlorobimane (MCB) assay, MCB is conjugated with GSH to form a fluorescent glutathione MCB adduct. DL-buthionine-[S,R]-sulfoximine was used as a control for complete GSH depletion. The MCB-GSH was analyzed using a fluorescence microplate reader with an excitation of 385 nm and an emission of 485 nm (Molecular Devices Gemini XS, Molecular Devices, Sunnyvale, CA, USA). The LDH activity was determined using a probe purchased from Promega (Madison, WI, USA) measuring absorption at 490 nm on a microplate reader (Spectromax plus 384, Molecular Devices, Sunnyvale, CA, USA).

Genotoxicity Test

The SOS chromotest is a bacterial genotoxicity test developed as an alternative to the Ames test.[40,41] This test was validated in a number of studies and evaluated for a number of potential mutagens.[40,41] The test is described in detail in our previous work.[56,57]

Briefly, *Escherichia coli* strain PQ 37 (obtained from Pasteur Institute, Paris, France) containing a fusion gene of a ß-galactosidase (ß-Gal) gene (*lacZ*) with a DNA damage "SOS" response gene (*sfiA*) was used in this assay. Activation of the SOS repair system by genotoxic agents is measured by photometric determination of the ß-Gal enzyme activity. To estimate for total protein synthesis, alkaline phosphatase (AP) production was measured. Induction factor, defined as a ratio of β-Galactosidase and alkaline phosphatase, was employed for the presentation of data.

RESULTS AND DISCUSSION

In the framework of this project, the following tasks were completed:

1) In vitro toxicity assays, more closely approximating the human respiration system response by employing murine macrophages, were performed on fractions extracted with subcritical water from diesel exhaust and wood smoke PM and ambient Pittsburgh PM influenced by coke emissions. Results confirmed the feasibility and potential of human bronchial epithelial cells for toxicity evaluation.

2) The genotoxicity of three ambient PM samples obtained from Dr. Allen Robinson (Carnegie Mellon University at Pittsburgh, PA) was evaluated.

3) The genotoxicity of two diesel exhaust PM collected with and without presence of an oxidizer (diethyl carbonate) was studied. The samples were obtained in collaboration with Dr. Artur Braun (Consortium for Fossil Fuel Science at University of Kentucky, Lexington, KY).

4) The article entitled "Toxicity of wide-range polarity fractions from wood smoke and diesel exhaust particulate obtained using hot pressurized water" was accepted for publication in the peer-reviewed journal Environmental Toxicology and Chemistry (Vol. 23, No. 9, 2004).

5) The results were presented as a poster at the 43rd Annual Meeting of the Society of Toxicology in Baltimore, MD, March 2004. In April 2004, the data were presented at the 14th Annual Meeting of the Society of Environmental Toxicology and Chemistry (SETAC) Europe in Prague, Czech Republic.

Oxidative Stress of Wood Smoke PM, Diesel Exhaust PM, and Ambient Pittsburgh PM Influenced by Coke Emissions

The antioxidant defense of a cell is disturbed by the depletion of GSH, by far the most abundant nonprotein thiol in eukaryotic cells, ranging in concentration from 0.1–10 mM in vivo.[58,59] Both upregulation and depletion of GSH can be associated with oxidative stress because GSH levels in the cells are dynamic. Massive GSH depletion occurs in the presence of high concentrations of samples with electrophilic centers or toxicants causing the production of ROS such as superoxide, hydrogen peroxide, and hydroxyl radical. Upregulation of GSH may be the first step in cell defense, when the cell responds to the first sign of oxidants by increased production of GSH.

Thus the potential oxidative stress was evaluated on the basis of GSH depletion and upregulation by exposing **murine macrophages (RAW 264.7)** to PM fractions (12 h). Intracellular GSH determination was performed with a MCB assay, MCB is conjugated with GSH to form a fluorescent glutathione MCB adduct. DL-buthionine-[S,R]-sulfoximine was used as a control for complete GSH depletion. All toxicity assays were performed in a 96-well plate format. Parallel to GSH depletion, cytotoxicity was monitored using LDH. Figure 1 shows GSH depletion and upregulation by different-polarity fractions of three different PM samples.

Figure 1 shows GSH depletion and upregulation by different-polarity fractions of three different PM samples. Although only selected data are shown, a dose response curve was

generated for each PM fraction in quadruplicate on four independent days. As with macrophages, the GSH upregulation/depletion assay was also performed with **human bronchial epithelial cells** (**BEAS-2B)**, cells which are expected to be more exposed to PM than alveolar macrophages.[22] Figure 2 demonstrates the comparable GSH depletion by wood smoke PM fractions in the presence of both cell lines. Both midpolar and also the expected nonpolar fractions of wood smoke PM depleted GSH and thus exposed the cells to the oxidative stress. The oxidative stress induced by nonpolar fractions may be related to frequently studied PAHs. The major components, previously determined by GS/MS, in midpolar fractions are oxyPAHs of methoxyphenols (syringol dimers) and three-ring PAHs (Figure 3).[56,57] To evaluate which of those compounds could potentially cause oxidative stress, we have determined the GSH depletion/upregulation in the presence of pure standards (Figure 4). While nonoxygenated PAHs did not deplete GSH, some of the quinonic PAHs depleted GSH, suggesting they may contribute to oxidative stress in wood smoke PM.

In contrast to wood smoke PM, not only nonpolar and midpolar fractions but also polar fractions (50°C and 100°C) of diesel exhaust PM contributed to GSH depletion. Kawamura et al. has previously reported that dicarboxylic acids may be extracted from diesel exhaust PM by 50°C water.[60] Thus dicarboxylic acids and similar species may exhibit oxidative stress. The GSH depletion in the 200°C fraction may correspond to nitropyrene previously determined in those fractions by GC/MS.[56,57]

In contrast to wood smoke and diesel exhaust PM, upregulation of GSH was observed in polar and midpolar fractions of the ambient Pittsburgh sample influenced by coke emissions.

Further study is necessary to find out which components are responsible for oxidative stress. Nevertheless, the aim of this work was to evaluate whether frequently neglected polar fractions of PM are of toxicological importance. Therefore, we have accomplished our proposed work and demonstrated the necessity of further study of polar and midpolar PM constituents.

The Genotoxicity of Ambient PM Samples Collected in Pittsburgh

The direct genotoxicity of PM collected in Pittsburgh by the research group of Dr. Allen Robinson (Carnegie Mellon University) was studied. Figure 5 shows comparable DNA damage caused by both Pittsburgh ambient PM samples. The PM collected in a highway tunnel did not exhibit significant genotoxicity. The lack of genotoxicity of the highway tunnel PM may be due to lower loading on the filter, and thus lower PM mass extracted and toxicologically evaluated. To determine whether the genotoxicity is due to the same PM constituents in both PM samples, we have determined genotoxicity of wide-range-polarity fractions of those samples (Figures 6 and 7). The dissimilarity in genotoxic profiles of both PM samples suggests that different constituents are responsible for the genotoxicity. While for ambient Pittsburgh PM the polar (25°C) fraction was the most genotoxic, in coke-influenced ambient PM the polar and midpolar fractions (50°–200°C) were of higher genotoxicity. The DNA damage caused by polar fractions (extracted at 25°C and 100°C) of ambient Pittsburgh PM is in contrast to previous work on diesel exhaust PM, where the genotoxicity was shown only in midpolar fractions (150°–200°C). This suggests that in PM samples of various origins, different constituents may contribute to the genotoxicity.

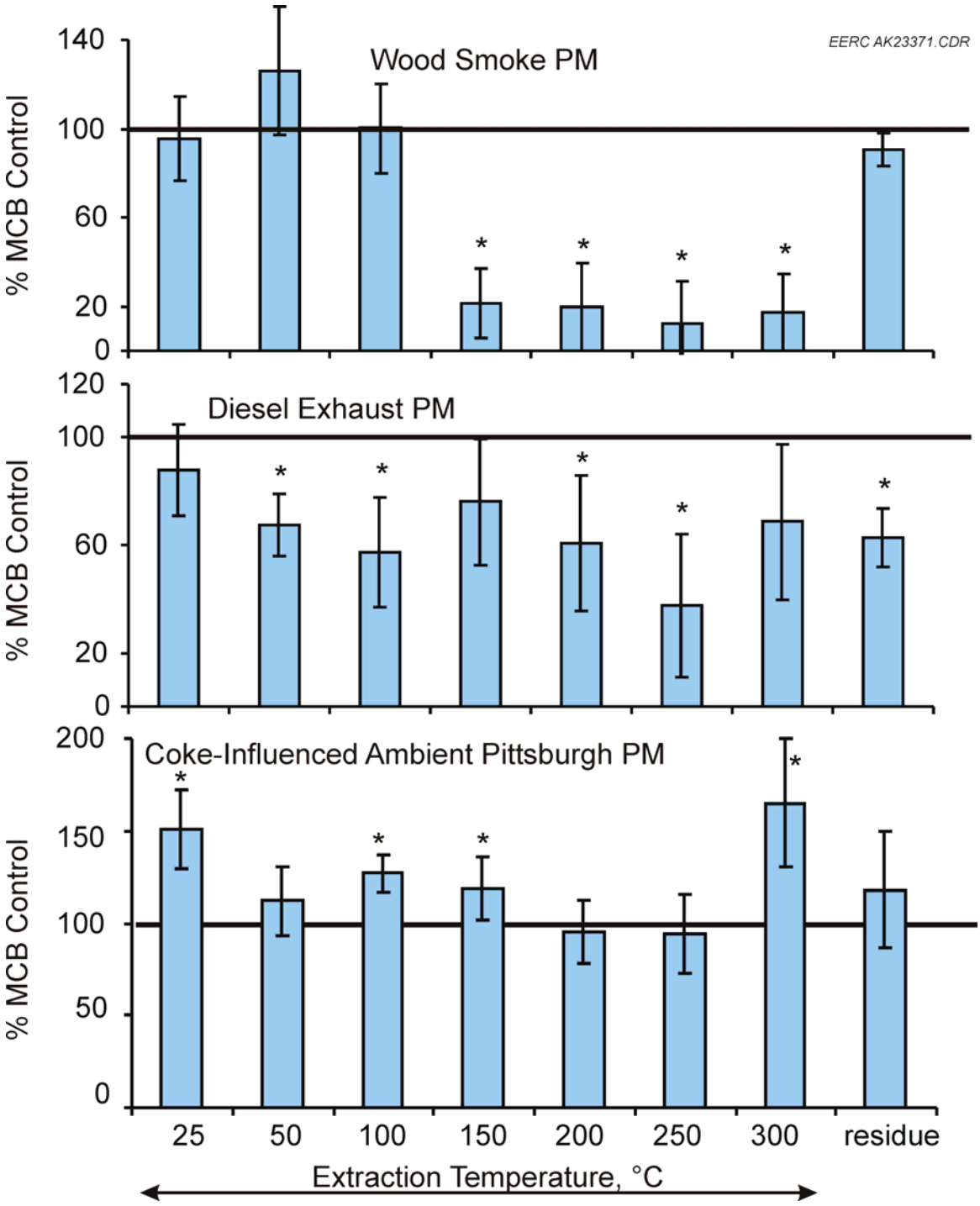

Figure 1. Comparison of GSH depletion in RAW 264.7 cells by different-polarity PM fractions (50 µg/mL) obtained with hot pressurized water. The GSH depletion was measured as formation of fluorescent MCB–GSH adduct. The data presented are mean ± standard deviations of four quadruplicates performed on four different days. The ambient sample influenced from coke emission was obtained from Dr. Allen Robinson of Carnegie Mellon University in Pittsburgh, PA. * Indicates a significant increase in genotoxicity.

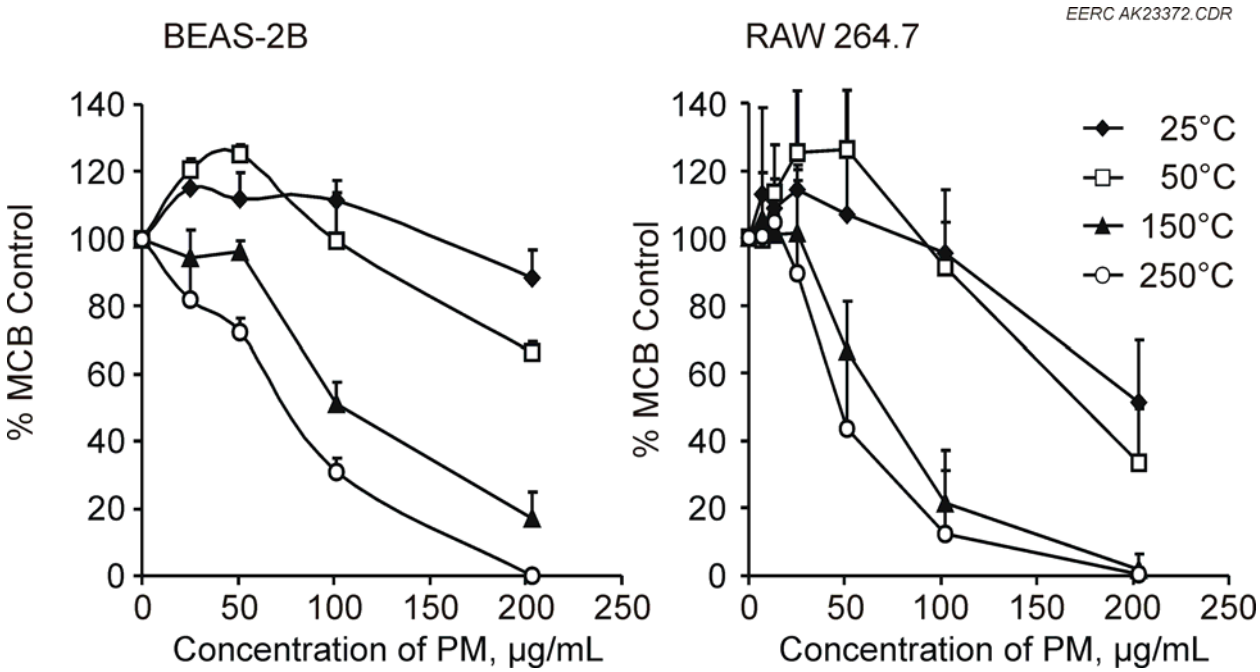

Figure 2. GSH depletion by wood smoke PM fractions obtained by hot pressurized water is comparable for human bronchial epithelial cells (BEAS-2B) and murine macrophages (RAW 264.7).

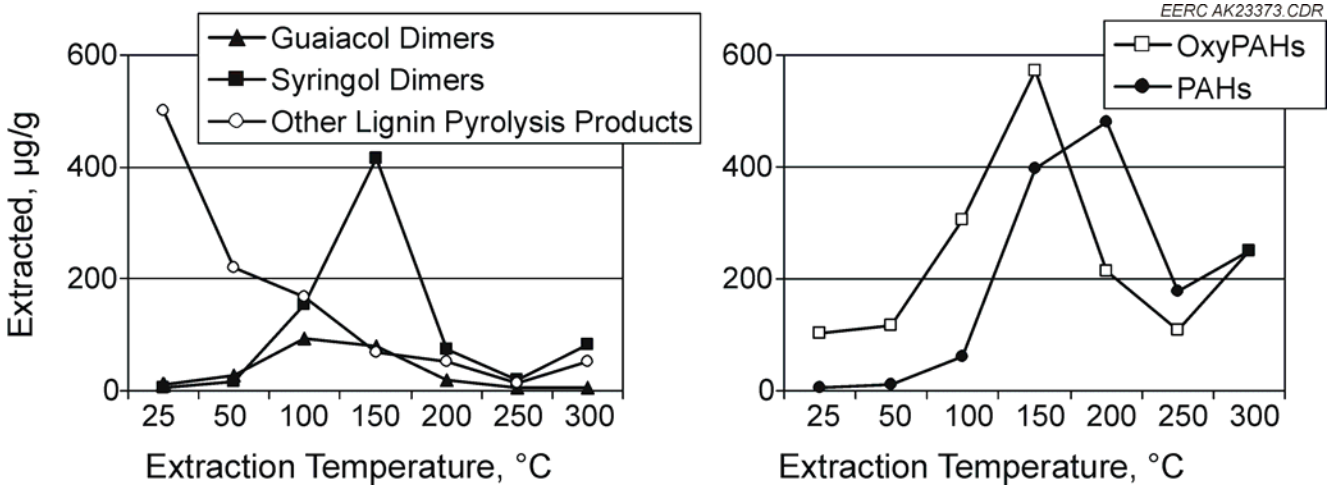

Figure 3. Major constituents of midpolar wood smoke fractions determined by GC/MS.

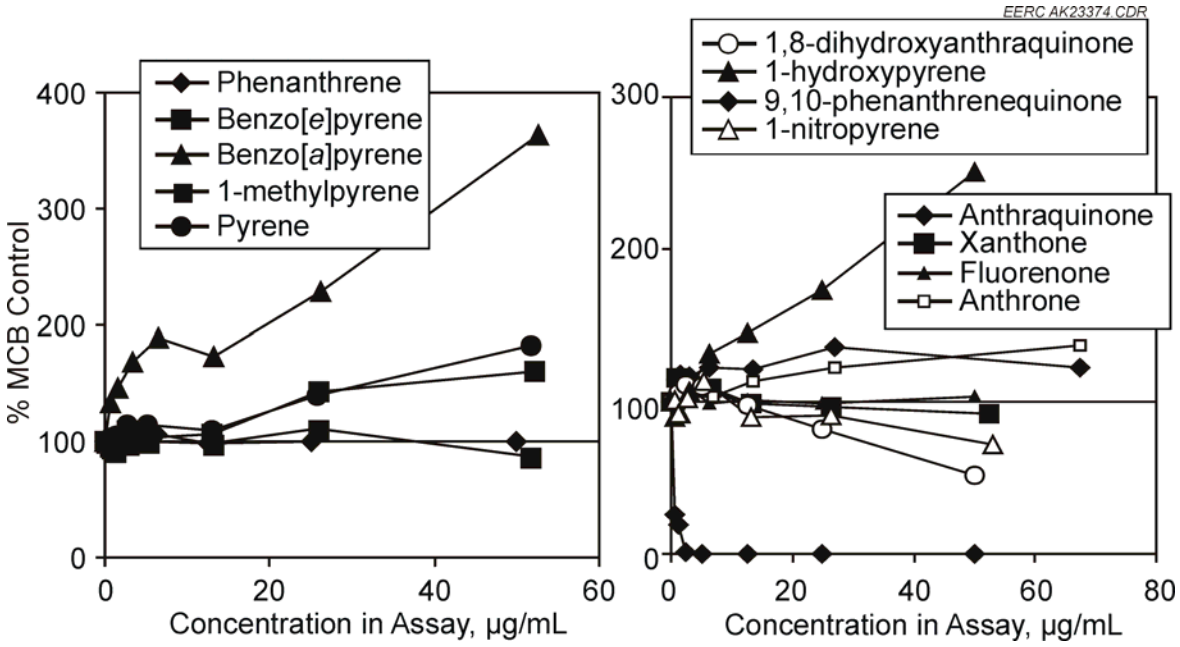

Figure 4. Upregulation/depletion of GSH in RAW 264.7 cells by PAHs (left) and OxyPAHs (right).

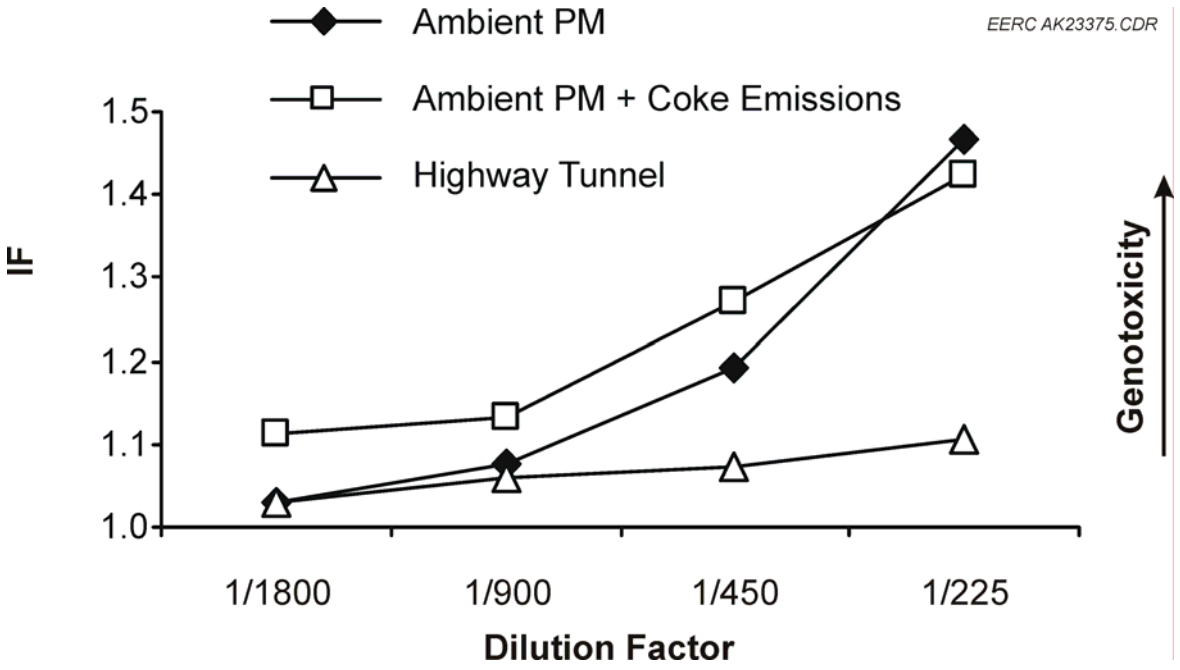

Figure 5. The direct genotoxicity determined using the SOS chromotest. Induction factor (IF) is defined as a ratio of β-galactosidase (a marker of DNA damage) and alkaline phosphatase (a marker of protein synthesis). The data presented are means of two triplicate experiments, the relative standard deviations were <5%.

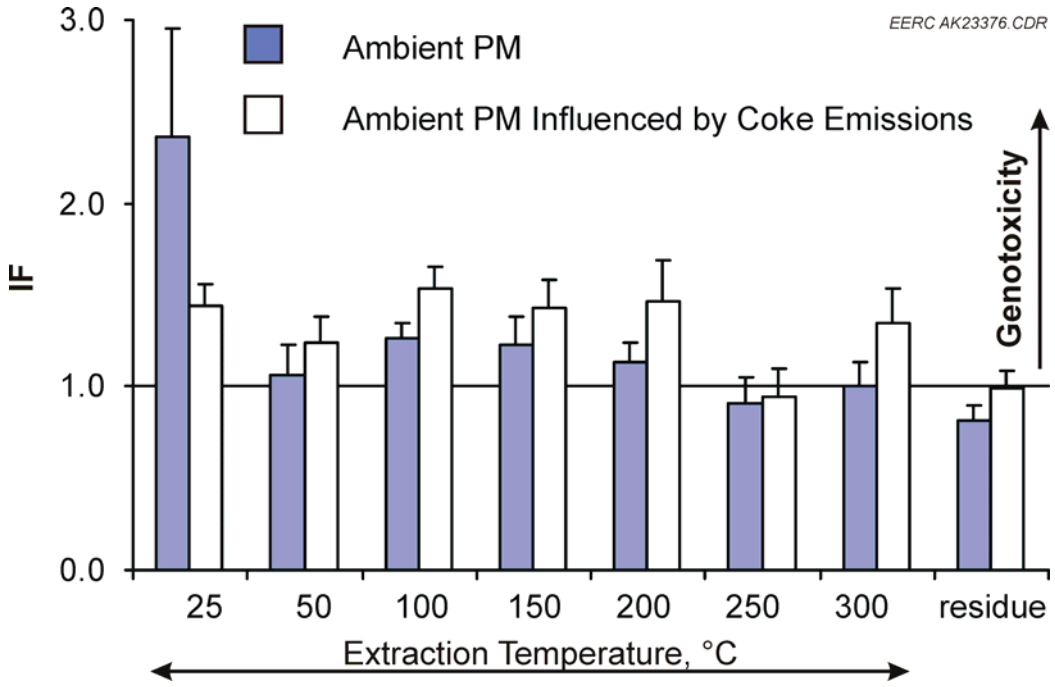

Figure 6. The direct genotoxicity of a wide-polarity range of fractions obtained from ambient Pittsburgh PM (dilution 1/100) with and without influence of coke emissions. Induction factor is defined as a ratio of β-galactosidase (a marker of DNA damage) and alkaline phosphatase (a marker of protein synthesis). The data presented are means of two triplicate experiments.

The Genotoxicity of Diesel Exhaust PM Generated with and Without the Presence of an Oxidizer

We have obtained two diesel exhaust PM samples from Dr. Artur Braun (University of Kentucky). Interestingly, the results show that diesel exhaust PM generated with diethyl carbonate (DEC) as an oxidizer exhibits higher genotoxicity than the reference diesel exhaust PM (Figure 8). The genotoxicity in midpolar fractions of DEC diesel exhaust corresponds to previous findings in our model diesel exhaust PM, for which the genotoxicity was attributed to nitropyrene and other more polar nitropolyaromatics possibly generated during combustion in the presence of NO_x gases and PAHs. Only the 250°C fraction of reference diesel exhaust exhibited significant genotoxicity.

Article on Toxicity of Wood Smoke and Diesel Exhaust PM

The article entitled "Toxicity of wide-range polarity fractions from wood smoke and diesel exhaust particulate obtained using hot pressurized water" was accepted for publication in the peer-reviewed journal Environmental Toxicology and Chemistry (Vol. 23, No. 9, 2004), and proofs of the article are enclosed.

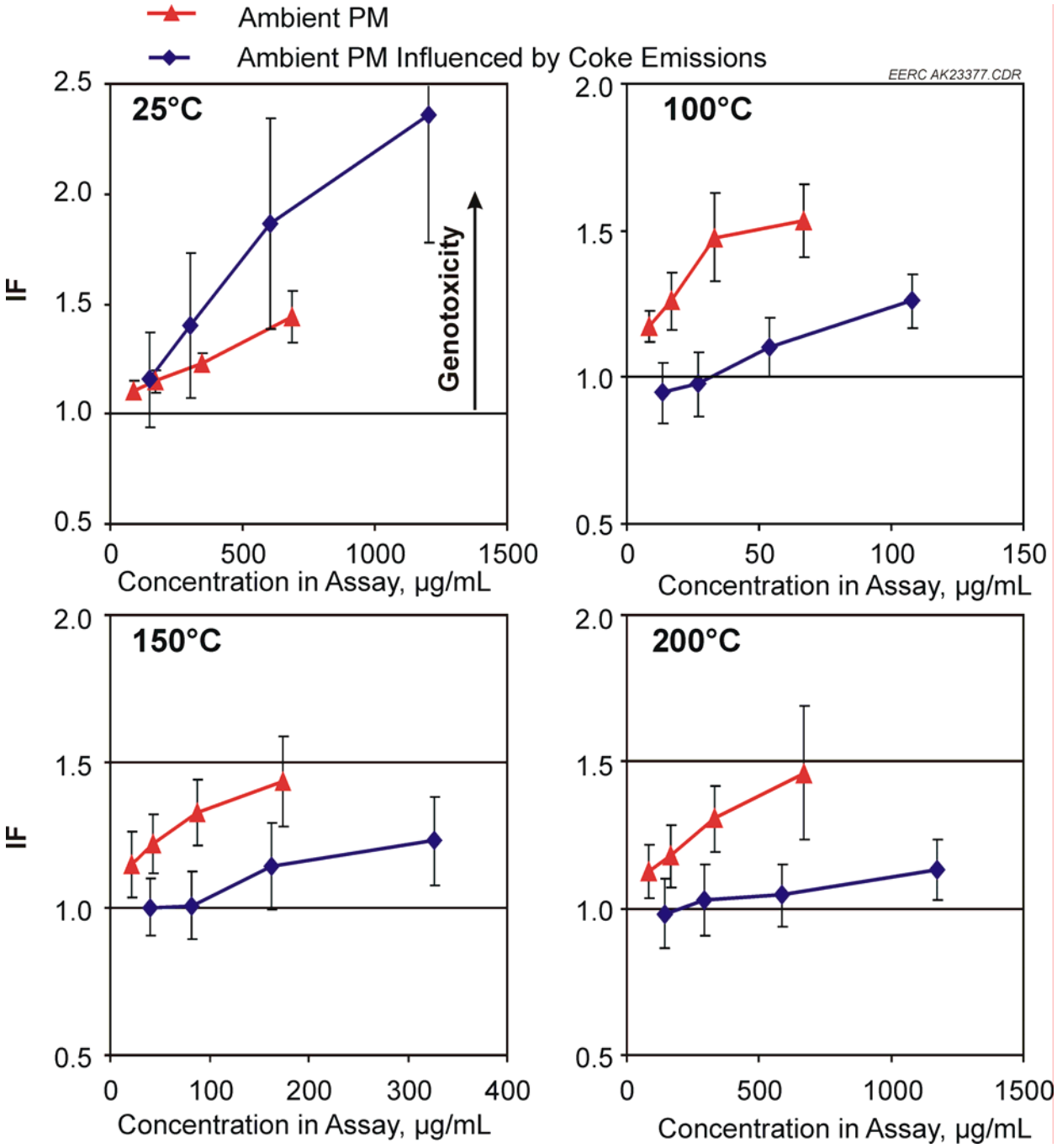

Figure 7. The dose-response direct genotoxicity curves of selected fractions obtained from ambient Pittsburgh PM (dilution 1/100) with and without influence of coke emissions. Induction factor is defined as a ratio of β-galactosidase (a marker of DNA damage) and alkaline phosphatase (a marker of protein synthesis). The data presented are means of two triplicate experiments.

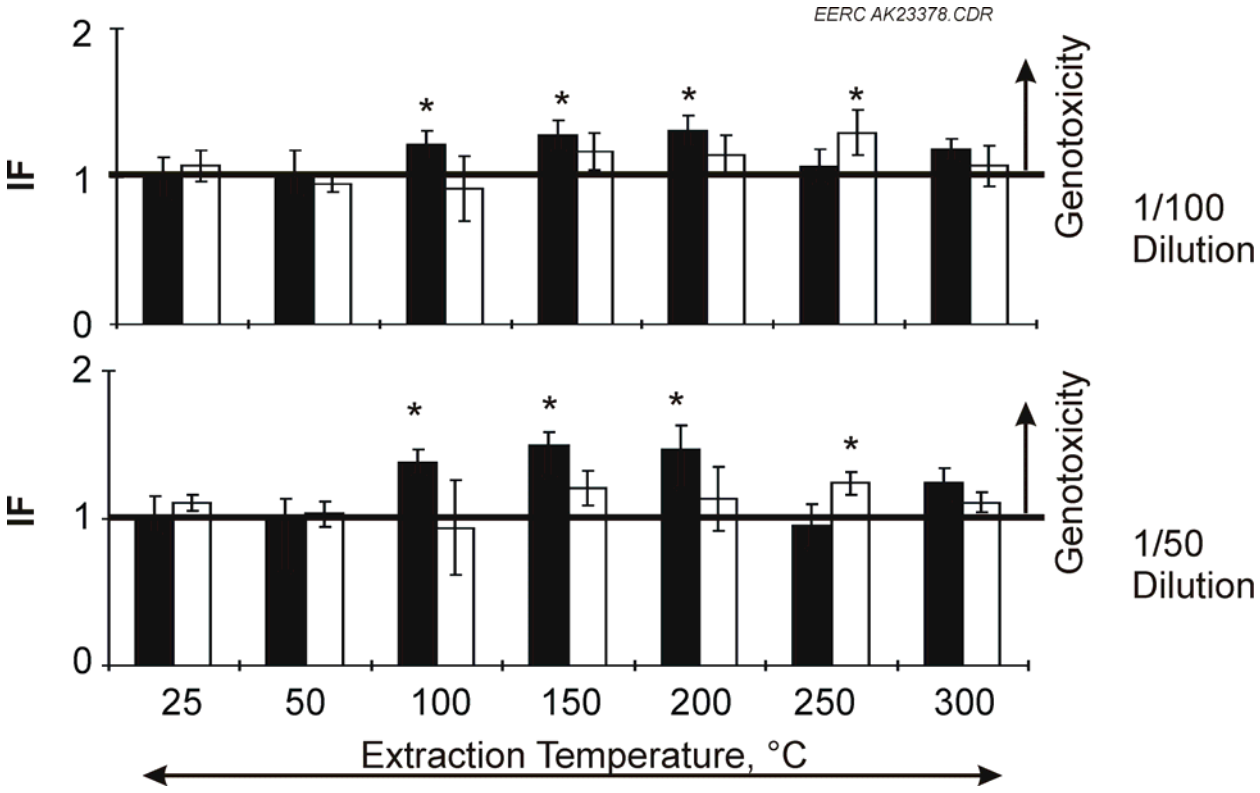

Figure 8. The direct genotoxicity of a wide-polarity range of fractions from diesel exhaust PM generated with (black bars) and without (clear bars) the presence of diethyl carbonate. IF is defined as a ratio of β-galactosidase (a marker of DNA damage) and alkaline phosphatase (a marker of protein synthesis). Two dilutions of the fractions are compared. *Significant increase in genotoxicity.

CONCLUSIONS

During this year, we have generated toxicological data that confirm our hypothesis showing the importance of polar PM constituents. The oxidative stress caused by the wide-polarity range of fractions of two model PM samples (wood smoke and diesel exhaust) was studied. In addition, the genotoxicity of fractions from ambient PM from Pittsburgh and two diesel exhaust PM samples as evaluated. We have confirmed the toxicological significance of polar PM fractions. In addition, we have shown that not one single class of compounds is responsible for the toxicological response observed. Finally, we have demonstrated that even samples of slightly different origin such as two diesel PM or ambient PM samples from Pittsburgh may have different toxicological impact. Thus the conditions at which PM was generated can significantly influence its toxicity.

Future study is necessary and the submitted proposals address the composition of the polar fractions and further toxicological effects, which can be linked to health effects.

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APPENDIX A

TOXICITY OF WIDE-RANGE POLARITY FRACTIONS FROM WOOD SMOKE AND DIESEL EXHAUST PARTICULATE OBTAINED USING HOT PRESSURIZED WATER PROOFS

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TOXICITY OF WIDE-RANGE POLARITY FRACTIONS FROM WOOD SMOKE AND DIESEL EXHAUST PARTICULATE OBTAINED USING HOT PRESSURIZED WATER

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Abstract—Epidemiological and toxicological studies correlate the adverse health effects of particulate matter (PM) with the available information regarding their chemical characterization, which has focused on nonpolar organics (e.g., polycyclic aromatic hydrocarbons [PAHs]), which are limited to 15 to 50% of total organic carbon. To study both polar and nonpolar species, we have employed hot pressurized (liquid) water for the fractionation of diesel exhaust and wood smoke PM. In agreement with the results of previous studies, nonpolar fractions from both PM samples showed strong cytotoxicity (cell viability decreased to 50–60%) corresponding to the presence of PAHs. Surprisingly, similar decreases in cell viability were also found in polar fractions (50°C) from both diesel exhaust and wood smoke PM. The midpolarity fractions (100–150°C) from wood smoke PM also displayed high cytotoxicity corresponding to methoxyphenols and oxy-PAHs. Although the midpolarity fractions from diesel exhaust PM showed no cytotoxicity, genotoxicity was found in the 150°C fraction, possibly corresponding to nitroaromatics. The present study demonstrates the suitability of hot pressurized water (as a single, nontoxic solvent) for the fractionation and toxicological characterization of wide-range polarity constituents of PM and, possibly, other environmental matrices.

Keywords—Wood smoke Diesel exhaust Hot pressurized water Cytotoxicity Genotoxicity

INTRODUCTION

Atmospheric particulate matter (PM) is a chemically complex matrix consisting of inorganic salts, metals, and elemental as well as organic carbon. In contrast to inorganic aerosols, only 15 to 50% of the organic carbonaceous particulate mass has been characterized [1–3]. The recovered organic species, so-called ''extractable and elutable organic matter,'' are limited by their extractability with organic solvents and their capability to be eluted using gas chromatography (GC) with respect to their thermal stability and molecular weight [1,2].

Besides the chemical characterization, the detrimental effects of aerosol PM are evaluated through correlation of the aerosol composition with the results of epidemiological studies and/or in vivo inhalation studies [4–6]. To evaluate the toxicity of different PM components, fractionation with a series of different organic solvents, usually coupled with in vitro toxicity tests, is employed [7–13]. Frequently, a lower-polarity solvent, such as methylene chloride, is employed for the recovery of crude extract, whereas the fractionation is performed using a solvent of stronger polarity, such as methanol [7–10]. Only a few studies have performed the extraction directly with a series of different-polarity solvents [11–13].

The advantage of hot pressurized (liquid) water (i.e., hot water under sufficient pressure to maintain it in the liquid state) is in the ability to extract a broad range of low- to high-polarity compounds [14,15]. A number of extraction studies using steam and supercritical water (temperature, >374°C; pressure, >221 bar) have been reported. However, hot liquid-phase (subcritical) water has only recently received attention, despite the fact that a much broader range of polarities can be achieved with hot pressurized water than with either steam or supercritical water [14,15]. The extraction capability of water is based on a change in its polarity by a simple temperature increase under low pressure (to maintain the liquid phase). Heating water from 25 to 300°C decreases its polarity, expressed as a dielectric constant (ε), from 78 to 20 [16]. In contrast to hot pressurized water, a polar solvent, such as methanol, has a dielectric constant of 30.

The practical use of controlling the polarity of water with temperature is related to the ability to solubilize various organic compounds [17–19]. The solubility of nonpolar organics is elevated by simple temperature increases. For example, the solubility of benzo[*a*]pyrene increases by 3×10^5–fold when changing the temperature from 25 to 250°C [18]. Thus, the change in solubility with increasing temperature was exploited for sequential extraction of phenols, polycyclic aromatic hydrocarbons (PAHs), and alkanes from petroleum waste sludge [20]; of oxygenated species (i.e., compounds similar to organics in wood smoke PM) and of nonpolar monoterpenes from savory [21].

The aim of the present study, therefore, was to evaluate the use of hot pressurized water for the extraction and fractionation of organic PM, to estimate the importance of the resulting fractions with two in vitro toxicity tests, and to compare the toxicity of the fractions to GC/mass spectrometry (GC/MS) characterization. The extraction/fractionation was evaluated using two common PM samples: Wood smoke PM and diesel exhaust PM, representing polar and nonpolar matrices, respectively.

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MATERIALS AND METHODS

Sample material

Bulk diesel exhaust PM was collected from the exhaust pipe of a diesel bus, homogenized, and stored frozen until use. Bulk wood smoke PM was collected from a chimney that vented an airtight wood stove burning a mix of hardwoods [22]. Fifty-milligram portions of each material were used for all extraction experiments.

Hot pressurized water extraction/fractionation

The term *hot pressurized water fractionation* corresponds to a sequential collection of fractions with increasing temperature. The total water extract, used for comparison with organic solvent extracts, was obtained by combining all fractions from the fractionation.

The hot pressurized water extraction was performed in an apparatus previously described in detail [18]. In brief, the extraction system consisted of an ISCO model 100D syringe pump (ISCO, Lincoln, NE, USA) delivering water at a constant flow rate of 0.5 ml/min to a preheating coil and extraction cell mounted in an HP 5890 GC oven (Hewlett-Packard, Wilmington, DE, USA). At the outlet of the extraction system, outside the oven, a HIP Model 15-11AF1 valve (High Pressure Equipment, Erie, PA, USA) was placed to maintain the system pressure, ensuring that water was in the liquid state at all temperatures. The outlet valve was heated (50–150°C) to prevent precipitation of extracted material. The extract was transferred from the outlet valve by a stainless-steel tube (length, 10 cm; inner diameter, 1.57 mm) to a collection vial that was cooled with ice to prevent sample loss.

Water extractions were carried out in a 3.47-ml, supercritical fluid extraction cell (length, 50 mm; inner diameter, 9.4 mm; Keystone Scientific, Bellefonte, PA, USA) equipped with 0.5-μm frits. The water (high-performance liquid chromatographic grade; Fisher Scientific, Pittsburgh, PA, USA) was purged with nitrogen to remove dissolved oxygen before the extraction. The extraction procedure started by pressurizing the system with water at a flow rate of 1 ml/min to approximately 50 bar (3 min). At this time, the water flow rate was set to 0.5 ml/min, the outlet valve was opened, and the collection of the eluent began (time = 0). To obtain different-polarity fractions, the extraction was performed sequentially at temperatures of 25, 50, 100, 150, 200, 250, and 300°C. Each temperature was held for 30 min, at which time the collection vial was replaced and the system heated to the next higher temperature (requiring 30–90 s). The pressure was first held at 50 bar, and at higher temperatures (>150°C), the pressure was increased to maintain water in the liquid state (see Fig. 1 for experimental conditions). To describe obtained fractions, we differentiate the polarity of fractions obtained by hot pressurized water based on the dielectric constant (Fig. 1). Each fraction was collected into 5 ml of water in a preweighed collection vial. After each extraction, the system was washed with water heated from 25 to 325°C (100 bar) and at 325°C (5 bar) with steam. The system was also washed in sequence with 5 ml of acetone, methylene chloride, and acetone again.

Solvent extraction

Hot pressurized water extraction was compared to extractions employing two different-polarity organic solvents: nonpolar *n*-hexane (Optima; Fisher Scientific) and polar methanol (Optima; Fisher Scientific). Fifty-milligram portions of PM were sonicated once for 18 h, followed by an additional three sonications for 30 min with 10 ml of solvent. After each sonication, the solvent extracts were filtered through a 0.45-μm Teflon® filter and combined.

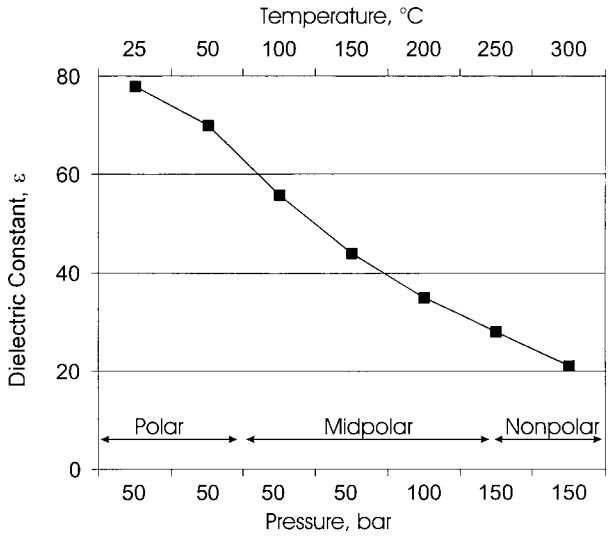

Fig. 1. Operating conditions of hot pressurized water fractionation and corresponding dielectric constant [16]. The pressures were selected to ensure that water remained in the liquid state. Based on the dielectric constant (ε), polarity ranges were defined for different temperature fractions.

Toxicity tests

Toxicity tests were performed on organic solvent extracts, combined water fractions, and individual water fractions. Toxicity tests were performed on nitrogen-dried extracts redissolved in dimethyl sulfoxide (DMSO) and Hanks' balanced salt solution (HBSS; Fisher Scientific). The DMSO was employed to transfer PM into aqueous suspension. The DMSO may act as a radical scavenger; therefore, it may affect PM toxicity [23]. Thus, we limited the concentrations of DMSO to 1% (v/v) in the toxicological assays. No effects of DMSO (1% [v/v]) were observed. We have also verified that the dried organic solvent residues (solvent blanks) did not exhibit a toxic response.

Cell viability

The cytotoxicity test measured the viability inhibition of mammalian cells (African green monkey kidney cells designated COS-1 [CV-1 cells with origin-defective mutants of SV40]) [24]. The cells were grown in tissue-culture flasks (75 ml) in Dulbecco's modified Eagle media (Fisher Scientific) with 10% (v/v) fetal calf serum and 1% (v/v) penicillin and streptomycin in a 37°C incubator. The cytotoxicity tests were performed on two independent days (each time in quadruplicate) on 96-well microplates in a 95% O_2, 5% CO_2 atmosphere. Based on a two-way analysis of variance (ANOVA), fractions showing significant cytotoxicity ($p < 0.0001$) typically showed less than 5% variance for experiments performed on two different days. On day 1, 10,000 cells per 180 μl of culture were plated to each well. On day 2, 20 μl of sample diluted in tissue-culture media (adjusted to pH 7) were added, so the final concentration of DMSO in tissue culture was 1% or less. After 24 h, cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyl tetrazolium bromide (MTT) assay [25,26]. The results were analyzed at an absorbance of

570 nm using a microplate reader (Spectromax Plus 384; Molecular Devices, Sunnyvale, CA, USA).

The MTT assay is based on the reduction of MTT by cellular oxidoreductase enzymes. Thus, viable cells give blue formazan salt, whereas damaged cells do not. The percentage cell viability was calculated as cells treated with the PM fractions normalized to untreated cells (exposed only to DMSO/HBSS solution). The untreated control, killed with 0.5% (v/v) Triton X-100 (Sigma Aldrich, Steinheim, Germany), was used as a colorimetric blank.

SOS Chromotest

The SOS Chromotest is a bacterial genotoxicity test [27,28] developed as an alternative to the Ames test [29]. This test was validated in a number of studies and evaluated for a number of potential mutagens [27,28,30]. *Escherichia coli* strain PQ 37 (obtained from Pasteur Institute, Paris, France) containing a fusion gene of a β-galactosidase (β-Gal) gene (*lacZ*) with a DNA-damage SOS response gene (*sfiA*) was used in this assay. Activation of the SOS repair system by genotoxic agents is measured by photometric determination of the β-Gal enzyme activity. To estimate for total protein synthesis, alkaline phosphatase (AP) production was measured. The modified procedure for the SOS Chromotest assay was based on previously published procedures [27,28,30,31]. The tester strain (180 μl of optical density OD_{600} = 0.05) prepared according to the method of Miller [31] and 20 μl of samples (four dilutions in 0.9% NaCl) were introduced into 2-ml polypropylene tubes, which were incubated with shaking for 2 h at 37°C. After incubation, two portions (80 μl) of each sample were withdrawn and placed into microplate wells. Half the samples were used for determination of AP activity and the other half for determination of β-Gal activity [27,28,30,31].

The procedure with metabolic activation was performed as previously described using rat liver microsome mixture S9 induced with Aroclor 1254 (In Vitro Technologies, Baltimore, MD, USA) added to the culture before incubation with samples [27–29].

The samples were kinetically read on the microplate reader at 420 nm (37°C) from 0 to 30 min at 5-min intervals. For the data evaluation, β-Gal and AP formations were expressed as slopes for the time interval of 10 to 30 min (linear range), which corresponds to a simplified calculation of enzyme units [31]. The β-Gal and AP activities were calculated as a ratio of slopes (S) in the presence and absence of active material, or $S(C)/S(C = 0)$, where C is the concentration of the extracted material in the assay. Mutagenic activity can be expressed as β-Gal activity, but because β-Gal can be decreased as a result of protein synthesis inhibition, an induction factor was calculated as a ratio of β-Gal and AP activity [27,28,30]. The induction factors for control compounds 4-nitroquinoline oxide (0.7 μM) and, with S9 activation, benzo[*a*]pyrene (20 μM) were 3.2 and 1.4, respectively. The AP activities for 4-nitroquinoline oxide (0.7 μM) and, with S9 activation, benzo[*a*]pyrene (20 μM) were 0.85 and 0.95, respectively. The genotoxicity tests were performed on two independent days in quadruplicate each time.

Chemical characterization

Total organic carbon (TOC) analyses were performed directly on water extracts obtained by extraction of 50 mg of PM using U.S. Environmental Protection Agency Method 415.1.

To perform the GC/MS analysis, aqueous fractions of PM were extracted with methylene chloride employing U.S. Environmental Protection Agency Method 625 for base/neutrals and acids extraction. Before the extraction with methylene chloride, surrogate standards were added to each water fraction. The surrogate standards used were phenol-d_5, nitronaphthalene-d_7, pyridine-d_5, naphthalene-d_8, chrysene-d_{12}, and dichlororesorcinol. To extract bases and neutrals, the pH of the water fractions was adjusted to greater than 11 with 1 M NaOH. Then, each hot pressurized water fraction was extracted by shaking three times for 2 min with 10 ml of methylene chloride, which was collected and concentrated under a stream of nitrogen to 0.2 ml. To extract acids, the hot pressurized water fractions were acidified to a pH of less than 2 using approximately 9 M sulfuric acid and extracted with methylene chloride. The 1-chloronaphthalene was added to each methylene chloride extract as an internal standard before analysis.

The GC/MS analyses were performed using a Hewlett-Packard model 5890 GC with a Hewlett-Packard model 5972 MS in the full-scan mode (45–500 m/z) with electron-impact ionization. Chromatographic separations were accomplished with a DB-5 column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm; J&W Scientific, Rancho Cordova, CA, USA) with injections in the splitless mode. The oven temperature was held at 40°C for 0.2 min, followed by a 10°C/min gradient to 320°C and then held again for 20 min. To achieve sufficient sensitivity for analyses of PAHs and nitro-PAHs in diesel exhaust fractions, the GC/MS analyses were performed in selected ion monitoring mode. For nitro-PAHs, cool on-column injection was employed and the temperature program modified to start at 40°C, followed by a 35°C/min gradient to 100°C, then a 5°C/min gradient to 320°C, and then held for 10 min. The individual components in aerosol extracts were identified using computer matches to standard reference mass spectra of the National Institute of Standards and Technology (Gaithersburg, MD, USA) library and literature data [16]. The identification was confirmed by reference standards of PAHs, lignin pyrolysis products, phenols, and so on. Quantitative analyses of more than 100 organics were based on the calibration with compounds typical for diesel exhaust and wood smoke PM, such as PAHs, nitro-PAHs, oxy-PAHs, phenols, aromatic acids, syringol, and guaiacol derivatives. For certain compounds, such as lignin pyrolysis products, not all standards were available; therefore, the response factor of a similar compound was used. For each class of compounds, at least three different standards were employed. For example, syringol was used for a tentative quantitation of dimethoxyphenol derivatives, syringaldehyde was used for syringol derivatives containing the aldehydic group, and allyl syringol was employed for higher-molecular-weight syringol derivatives.

Statistical analysis

Statistical comparisons were performed with one-way or two-way ANOVA when appropriate using Prism software (GraphPad, San Diego, CA, USA). Results are expressed as the mean ± standard deviation.

RESULTS

Comparison of hot pressurized water and organic solvent extraction

For the PM analytical and toxicological characterization, different organic solvents are employed [1–3]. The extraction

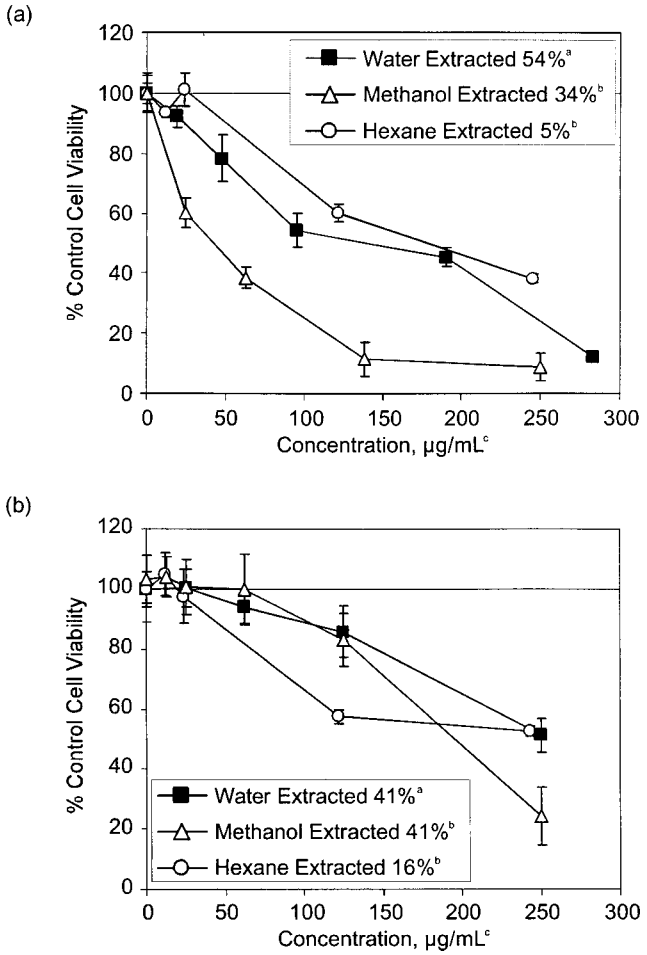

Fig. 2. Cytotoxicity (mammalian COS-1 cells) of hot pressurized water versus organic solvent extracts from (**a**) wood smoke and (**b**) diesel exhaust particulate matter (PM). Results are shown as the mean ± standard deviation (n = 4). [a]Hot pressurized water extracts were obtained by combining all fractions (25–300°C); 54% and 41% (w/w) of material were extracted from 50 mg of wood smoke and diesel exhaust PM, respectively. [b]Solvent extractions were performed by sonication (once for 18 h and three times for 30 min each time); the percentage (w/w) corresponds to material extracted from 50 mg of each PM. [c]Concentration was calculated as the mass of the extracted material diluted in the cell culture.

capability of hot pressurized water in contrast to that of organic solvents was compared on the basis of mass in the extracts, cell viability tests, and GC/MS analysis. Two different-polarity organic solvents, nonpolar hexane (ε = 2) and polar methanol (ε = 30), were compared with the hot pressurized water extraction (ε = 20–78) (Fig. 1).

For wood smoke PM (a polar matrix because of the high content of oxygenated species) (Fig. 2a), water extracted substantially more mass (54% of the total PM sample) than methanol (34%) or hexane (5%). Thus, the mass of material extracted increased with the polarity of the solvent (Fig. 2a). Methanol extracted a complex mixture of organics, including polar species, such as syringol and guaiacol derivatives, as well as less polar syringol dimers and oxy-PAHs and nonpolar PAHs. Compounds identified in the methanol extract were also observed in the hot pressurized water extract. However (as shown later), hot pressurized water allowed for selective extraction of different-polarity components by temperature changes. The higher cytotoxicity of methanol extracts in contrast to those of hexane also indicates the contribution from

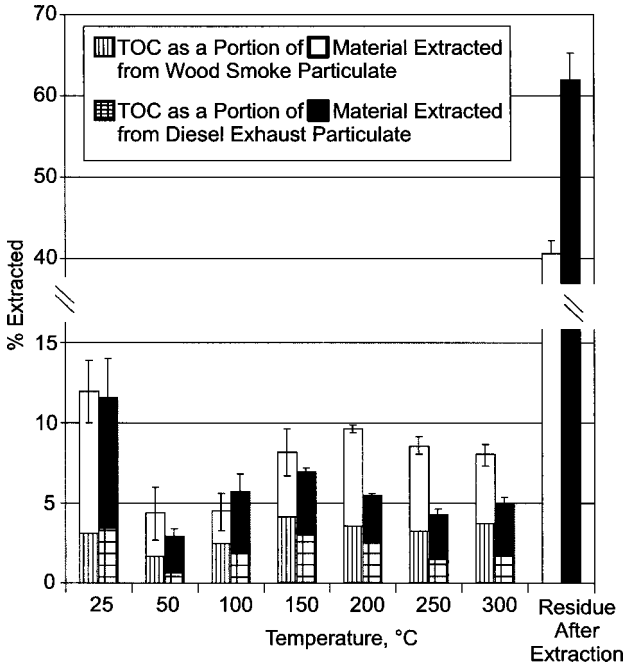

Fig. 3. Total organic carbon (TOC) shown as a portion of material extracted from wood smoke and diesel exhaust particulate matter in each hot pressurized water fraction (25–300°C). The standard deviations correspond to triplicate fractionation experiments.

polar components (Fig. 2a). The toxicity of wood smoke methanol extracts is also higher than that of hot pressurized water (Fig. 2a), but if the toxicity was related to the total mass recovered by each solvent, then the total toxicity of hot pressurized water would have to be corrected by a factor of 1.6 (the ratio between the mass of material extracted by water and by methanol). Thus, the same compounds may be responsible for toxicity in methanol and hot pressurized water extracts.

For diesel exhaust PM (a nonpolar matrix containing mainly alkanes and PAHs), both water and methanol extracted 41% of the PM mass, whereas hexane only extracted 16% (Fig. 2b). The discrepancy in toxicity profiles of different solvent extracts may be attributed to their different compositions. The total toxicity of extracts can be influenced by possible additive, synergistic, or antagonistic effects of the different classes of compounds present. Therefore, without further fractionations, the extracts are complex; consequently, it may be difficult to attribute toxicity to individual components.

Hot pressurized water fractionation and elemental characterization

The cytotoxicity and mass of extracted material in extracts obtained with organic solvent and hot pressurized water (described above) suggest a contribution from polar components. To find out which PM components have the strongest effect on toxicity, wood smoke and diesel exhaust PM samples were fractionated into seven fractions using hot pressurized water of a dielectric constant ranging from 20 to 78 (Fig. 1).

Initial characterization showed the mass of material extracted and the TOC content distributed over all fractions (Fig. 3). Based on good agreement between TOC and previously determined elemental carbon, we can say that extracted carbon is essentially all organic (http://www.netl.doe.gov/publications/proceedings/02/air_q3/Organic%20Aerosol.pdf). Still, the question remains as to the chemical composition of the

remaining extracted material (Fig. 3). The difference between the mass of material extracted and the TOC can be attributed, at least partially, to the oxygen in organic compounds. The mass of organic compounds is usually estimated by multiplying the TOC by a response factor that corrects for oxygen content in different matrices [2]. The response factors for wood smoke PM and diesel exhaust PM employed were 2.4 and 1.4, respectively [2]. Multiplying the TOC of wood smoke PM by 2.4 showed that all extracted material in hot pressurized water fractions corresponded to organic compounds. Based on the response factor of 1.4 for diesel exhaust PM fractions, not all the extracted material can be attributed to organic compounds. The mass of organic compounds in nonpolar fractions (200–300°C) corresponded to the extracted mass, but for midpolarity fractions of 50, 100, and 150°, only 50%, 60%, and 70% (w/w), respectively, were attributed to organics. The mass of organics in the 25°C fraction, estimated using the response factor of 1.4, was less than 10% (w/w) of the mass extracted.

The estimated lower quantity of organics in diesel exhaust PM may be caused by the presence of more polar compounds, which are not accounted for in the response factor, or by inorganic species in those fractions. This is supported by the previously reported, increased sulfur concentration of 17%, 14%, and 6% (w/w) in the 25, 50, and 100°C fractions, respectively (http://www.netl.doe.gov/publications/proceedings/02/air_q3/Organic%20Aerosol.pdf). In addition, previous data showed a higher content of metals, such as zinc, iron, and magnesium, in the 25°C fraction. Thus, the large portion of extracted diesel exhaust PM at ambient temperature may correspond to water-soluble metal salts, such as sulfates of zinc, iron, and magnesium.

Cytotoxicity of different-polarity fractions with respect to GC/MS analysis

The importance of different-polarity fractions extracted from PM samples was first evaluated with a mammalian cytotoxicity test (Fig. 4). For wood smoke PM, significantly increased cytotoxicity was found in fractions of 25 to 250°C, in contrast to the nonpolar fraction of 300°C ($p < 0.001$, two-way ANOVA). For diesel exhaust PM, the cytotoxicity was higher in nonpolar fractions (250 and 300°C) and, interestingly, in the polar fraction of 50°C ($p < 0.001$).

To define possible candidate toxicants, GC/MS analyses were performed on these fractions. The GC/MS characterization of wood smoke PM fractions (Fig. 5) showed selective hot pressurized water extraction of different classes of compounds. Increased concentrations of aromatic acids and their methyl esters, phenols, and lignin pyrolysis products (guaiacol, syringol derivatives, levoglucosan, and other methoxyphenols) were observed in the most polar fractions of 25 to 100°C (Fig. 5). Heavier-molecular-weight oxygenated species, such as syringol and guaiacol dimers, and oxygenated PAHs were found in the midpolarity fraction of 150°C. Finally, nonpolar PAHs were found in mainly 150 and 200°C fractions (Fig. 5). All those organic compounds may have contributed to the cytotoxicity of the particular fraction. None of the GC/MS-determined organics was preferentially extracted in the most toxic fraction of 100°C.

The cytotoxicity of diesel exhaust PM nonpolar (250 and 300°C) fractions corresponded to increased concentrations of PAHs (40 and 100 μg/g, respectively). It is interesting to note that the majority of PAHs were extracted from diesel exhaust PM at a higher temperature (300°C) than the majority from

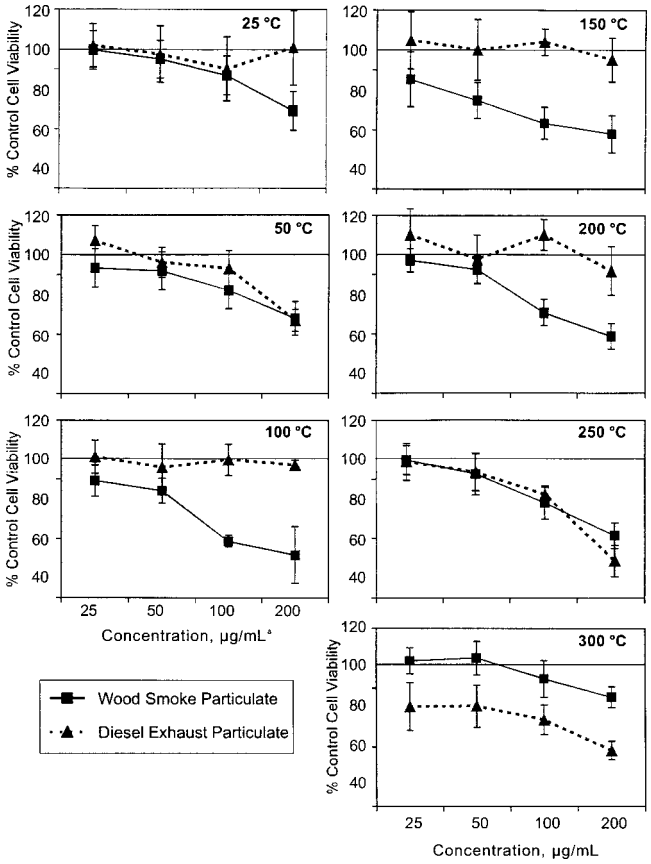

Fig. 4. The cytotoxicity of wood smoke and diesel exhaust particulate is dependent on the temperature of hot pressurized water fractionation. The results are shown as the average ± standard deviation of two experiments performed in quadruplicate ($n = 8$). For wood smoke particulate matter (PM) fractions, a one-way analysis of variance (ANOVA) indicated significant decrease in the cell viability ($p < 0.01$) in all fractions compared to the control (0 μg/ml of extracted material per assay). Two-way ANOVA showed wood smoke fractions of 25 to 250°C to be more toxic ($p < 0.001$) than the fraction of 300°C. For diesel exhaust fractions, one-way ANOVA indicated a significant decrease in cell viability ($p < 0.001$) compared to the control (0 and also 25 μg/ml of extracted material) for fractions of 50, 250, and 300°C. [a]Concentration was calculated as the mass of extracted material diluted in the cell culture (the initial mass of each PM was 50 mg; the mass extracted in each fraction is shown in Fig. 3).

wood smoke PM (150 and 200°C), possibly because water is less effective at displacing PAHs from soot-like matrices than from more polar matrices [32]. The cytotoxicity of the polar diesel exhaust fraction (50°C) might result from dicarboxylic acids previously found in diesel emissions [33].

Genotoxicity of diesel exhaust PM

The genotoxicity in hot pressurized water fractions was determined using the SOS Chromotest. No genotoxic response was observed in wood smoke PM extracts. In contrast, significant genotoxicity (based on the β-Gal activity) was observed in midpolarity fractions (50–250°C) of diesel exhaust PM extracts (Fig. 6a and b). For clarity, the comparison of different fractions is presented in Figure 6a using a single concentration of 200 μg/ml. These data are supported by the dose–response curves (25–200 μg/ml) shown in Figure 6b. The high genotoxicity in the 150, 200, and 250°C fractions may be attributed to nitropyrene in those fractions (0.7, 0.4, and 0.1 μg/g, respectively). Besides nitropyrene, we can as-

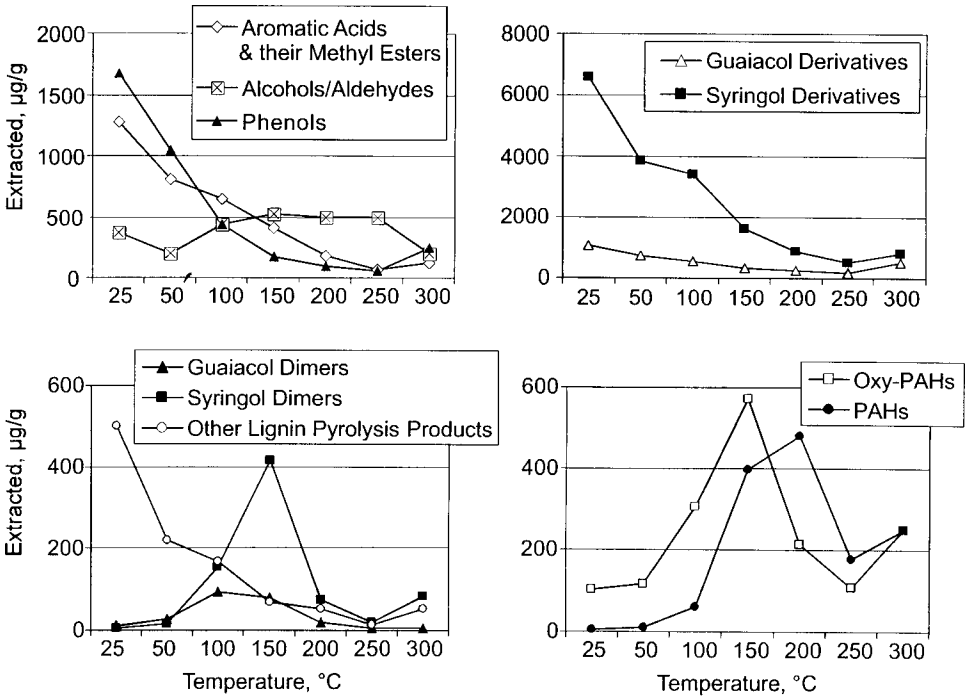

Fig. 5. The selective fractionation of organic compounds from wood smoke particulate matter (PM) using hot pressurized water fractionation. The organic compounds were determined using gas chromatography with mass spectrometry. Extracted = the mass of organics extracted per mass of PM (50 mg) used for the fractionation; PAH = polycyclic aromatic hydrocarbon.

sume the presence of and genotoxic contribution from other nitro-PAHs, for which a more sensitive analytical method would be needed [10]. The GC/MS analysis did not reveal a possible source of high genotoxicity in the 100°C fraction. This corresponds to the previously reported genotoxicity in polar extracts because of compounds more polar than nitro-PAHs [10].

The genotoxicity expressed as an induction factor includes a correction caused by the inhibition of total protein synthesis (AP activity) (Fig. 6a). The significant inhibition in AP activity in fractions from 25 to 150°C ($p < 0.01$) likely results from genotoxic damage to the AP gene, hence lowering the levels of AP protein. Alternatively, AP enzyme inhibitors may have been present in those fractions. Use of the induction factor allows for differentiation of genotoxicity in 150 and 200°C fractions ($p < 0.0001$, two-way ANOVA comparing induction factors).

Because certain organics are not directly mutagenic except after a metabolic activation, the tests were also performed with activation using rat liver microsome S9. Although hot pressurized water extracts contained PAHs, which are typical promutagens, no increase in genotoxic response was found after activation. This corresponds with previously reported data showing a lower genotoxic response in diesel exhaust extracts with the addition of S9 [34,35]. The possible explanation is that concentrations of promutagens, namely benzo[*a*]pyrene, are too low to be detected by the genotoxic assay (2 µg/g of benzo[*a*]pyrene).

DISCUSSION

The characterization of carbonaceous atmospheric PM is limited to the identification of nonpolar and slightly polar organics because of the use of organic solvents and GC/MS analysis. In the present study, we have demonstrated the suitability of hot pressurized water for fractionation of atmospheric PM into the range of low- to high-polarity fractions. This method allows for the replacement of a series of organic solvents by hot pressurized water. Moreover, significant toxicity was observed in frequently neglected polar fractions of both polar and nonpolar PM matrices.

Initial comparison of the total solvent extract's toxicity and the percentage of PM extracted (hot pressurized water, methanol, and hexane) indicated the extraction of a different range of components, which may contribute to the toxicity to the same or a different extent depending on their synergistic, additive, and antagonistic effects (Fig. 2). For example, the comparable toxicity of diesel exhaust extracts may suggest a similar composition for all extracts. However, this was rebutted by the high cytotoxicity of the diesel exhaust polar (50°C) fraction, possibly because of the presence of dicarboxylic acids [33] or other polar compounds that are not expected to be extracted by nonpolar hexane [10]. Thus, hexane may omit the extraction of polar compounds, and methanol and hot pressurized water might not extract some nonpolar compounds, such as alkanes [32]. Consequently, when correlating toxicological data with chemical composition of PM, it is important to take into account the sample preparation methods employed for toxicity and the analytical studies performed.

The advantage of hot pressurized water is in the fractionation of PM into the range of low- to high-polarity fractions using a single solvent in contrast to the typically used series of different-polarity organic solvents [11–13]. It is interesting to note that in only a few studies have a series of different-polarity organic solvents been used for the fractionation of PM [11–13]. In most fractionation studies, the samples were first extracted with slightly polar methylene chloride ($\varepsilon = 9$), and the crude extracts were then fractionated into different-polarity fractions, neglecting the fact that polar components might not be extracted during the initial extraction with methylene chloride [7–10].

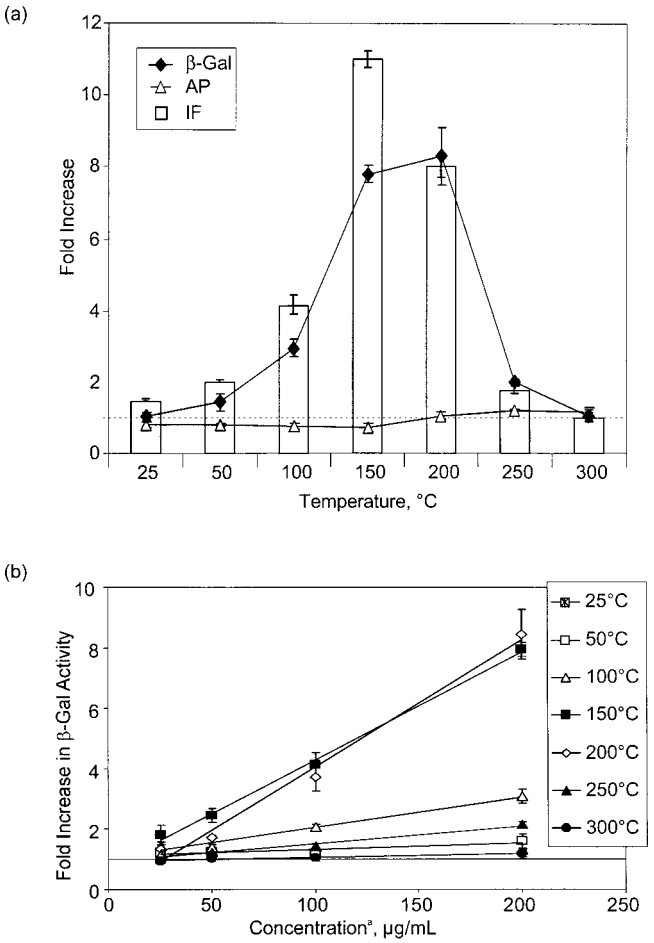

Fig. 6. Hot pressurized water fractionation selectively isolated genotoxic species from diesel exhaust particulate matter (PM). (**a**) The genotoxicity of 200 μg/ml of extracted material in cell culture was expressed as the fold-increase in β-galactosidase (β-Gal) formation and in induction factor (IF) based on the β-Gal to alkaline phosphatase (AP) ratio. The β-Gal activity was significantly higher in fractions of 50 to 250°C than in fractions of 25 or 300°C ($p < 0.005$, two-way analysis of variance [ANOVA]). The IF increase was significant for fractions of 25 to 250°C compared to the 300°C fraction ($p < 0.0001$, two-way ANOVA). The AP activity corresponds to the protein inhibition. One-way ANOVA (200 μg/ml) has shown significant AP inhibition in fractions of 25 to 150°C, in contrast to those of 200 to 300°C. [a]Concentration corresponds to the mass of organics extracted per mass of PM (50 mg) used for the fractionation.

The hot pressurized water allowed for fractionation and evaluation of the toxicity of different-polarity fractions with respect to GC/MS analysis. The highest cytotoxicity of the 100°C wood smoke PM fraction, in which no selectively extracted GC/MS-determined compounds were observed, may be attributed either to the synergistic effect of identified compounds or to the presence of compounds that could not be determined using GC/MS analysis of methylene chloride extracts. Therefore, the toxicity of individual compounds and defined mixtures of organics present in PM should be evaluated. Moreover, the chemical characterization of carbonaceous PM should be performed with a focus on polar species using other techniques, such as liquid chromatography with MS [36].

In contrast to organic solvents, water at ambient temperature extracted organic species as well as inorganic species, such as metal sulfates, for which health effects are still considered to be an unresolved issue [4,37]. Because of their high solubility in aqueous media, the components extracted at ambient temperature would be expected to have excellent bioavailability, resulting in adverse effects on the respiratory system.

The bioavailability of the atmospheric PM compounds to the respiratory system is another aspect to be considered in toxicological studies. In the present study, we have focused on the toxicity evaluation of different-polarity fraction compounds on an equal basis using a small amount of DMSO to ensure dissolution of all components. The next logical step for toxicity evaluation of PM is modeling real exposure to the respiratory system. The modeling of this exposure usually involves fast extraction of the PM with aqueous buffers and determination of toxicity in aqueous suspensions [37,38]. Still, it is important to note that although this approach, based on the bioavailability of PM components, is suitable for acute toxicity screening, it will likely neglect the identification of nonpolar compounds, such as PAHs.

In summary, the present study demonstrated the capability of hot pressurized water to fractionate different classes of compounds contributing to the total toxicity of PM, including water-soluble metal species and less studied polar components. With this method, the direct relationship between the different-polarity fractions obtained by a single solvent (water) and toxicity can be evaluated. Such direct evaluation cannot be performed when studying the toxicity of extracts obtained by a single organic solvent. Hot pressurized water fractionation may also be useful for the toxicological evaluation of other environmental matrices, such as soils and sediments.

Nevertheless, further studies are necessary to evaluate the bioavailability of the PM components in different models of the respiratory system. The present study indicates that more detailed toxicological testing and chemical characterization of carbonaceous PM should be performed with a focus on polar species.

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